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The effect of silymarin on prevention of hippocampus neuronal damage in rats with temporal lob epilepsy

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ABSTRACT

Background and Objective: Temporal lobe epilepsy is hallmarked with neuronal degeneration in some areas of hippocampus and mossy fiber sprouting in dentate area. Considering some evidences on neuroprotective and antioxidant activity of silymarin (SM), this study was undertaken to evaluate the preventive effect of this agent on structural changes in hippocampus of kainate-epileptic rats.

Materials and Methods: In this study, 32 male rats were divided into sham, SM-treated sham, epileptic, and SM-treated epileptic group. Rat model of epilepsy was induced by unilateral intrahippocampal administration of 0.8 µg kainic acid per rat. Rats received SM (100 mg/kg, i.p.) daily for 3 days before the surgery. Finally, brain sections were stained with Nissl and Timm methods.

Results: Induction of epilepsy was followed by a significant seizure and SM pretreatment did not lower seizure intensity. In addition, density of Nissl-stained neurons in CA3 and CA4 areas of hippocampus was significantly lower in epileptic rats versus sham ($p<0.05$) and SM pretreatment did not significantly prevented it in. Regarding mossy fiber sprouting, epileptic rats showed a higher degree of sprouting as compared to sham group ($p<0.01$) and SM pretreatment did not significantly lowered it.

Conclusion: SM pretreatment does not reduce seizure intensity, could not preserve hippocampal neurons and could not lower mossy fiber sprouting in kainate-induced epileptic animals.

1. Introduction

Epilepsy is a common neurologic disease around the world that is typified by recurrent intractable seizures (1). Temporal lobe epilepsy (TLE) is clinically the most common form of epilepsy that is instigated from the medial regions of temporal lobe such as amygdala and hippocampus (2, 3). Increased oxidative stress due to generation of excess reactive oxygen species (4) and inflammatory mediators (5) play important roles in cell damage in epileptic conditions. Kainic acid (kainate), an analogue of excitatory neurotransmitter glutamate, is usually used for inducing model of TLE in rodents like rats (6-10). TLE is rather resistant to current treatments (11, 12) and present anti-epileptic agents are rather ineffective or are associated with intolerable complications (10, 13). Thus, there is incraesing demand for design of new agents. Neuroprotective agents are currently suggested as promising substitutes for prevention and treatment of TLE (8, 9).

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Silymarin (SM) is a polyphenolic compound isolated from the fruits and seeds of the medicinal plant milk thistle (*Silybum marianum*), generally used for treatment of liver diseases (14) with antioxidant, anti-apoptotic, anti-inflammatory, and neuroprotective potentials (15-21). The anti-oxidant effect of SM is attributed to its potential to scavenge excess free radicals and augmentation of antioxidant system (22). SM could also inhibit microglia activation and lower level of inflammatory mediators and protect dopaminergic neurons against lipopolysaccharide-induced toxicity (23). In addition, several researches have shown protective potential of SM in multiple models of neuronal injury including focal cerebral ischemia and cerebral ischemia-reperfusion-induced brain injury in rodents (24, 25). Therefore, we tried to assess whether silymarin could prevent hippocampal neuronal damage in rats with temporal lob epilepsy.

2. Materials and Methods

All experiments of this study were done on male adult Wistar rats (200-240 g, n = 32) (procured from Pasteur's Institute, Tehran). The rats were kept three to four/cage in a temperature-controlled room under 12:12 light-dark cycle with food and water available *ad libitum*. All procedures involving animals were done in compliance with NIH guidelines for the care and use of laboratory animals. In this study, all efforts were applied to lower animals' sufferings.

Rats were divided into equal-sized sham operated (sham), Silymarin-treated sham-operated (sham+Silymarin), kainate, and Silymarin-treated kainate (kainate+ Silymarin) groups. For intrahippocampal injections, rats were anaesthetized with a solution of chloral hydrate (300-350 mg/kg), placed into the stereotaxic frame (Stoelting Co., USA) with incisor bar at 3.3 mm below the interaural line. The dorsal surface of the skull was exposed and a burr hole was drilled in the skull using the following stereotaxic coordinates according to the atlas of Paxinos and Watson (26): anteroposterior (AP), 4.1 mm caudal to bregma; 4.1 mm lateral to the midline (right side), and 4-4.2 mm ventral to dura. A 5 µl microsyringe filled with normal saline containing 0.2 µg/µl of kainate was placed over the burr hole and kainate solution was injected at a rate of 1 µl/min in order to induce experimental model of TLE. Kainic acid (kainate; Sigma-Aldrich, USA) was dissolved in cold normal saline just prior to operation. The sham group received an equivalent volume of normal saline at the same stereotaxic coordinates. The microsyringe was slowly withdrawn after 5 min and the rat scalp was sutured. The sham+Silymarin group received Silymarin (Sigma-Aldrich, USA) at a dose of 100 mg/kg/day starting 3 days before the surgery and the last treatment was 1 h before the surgery. Silymarin was dissolved in propylene glycol.

2.1. Behavioral evaluation of seizure activity

All animals were assessed for status epilepticus during the first 24 h post-surgery according to Racine's classification: 0, no reaction; 1, stereotypic mounting, eye blinking, and/or mild facial clonus; 2, head nodding and/or multiple facial clonus; 3, myoclonic jerks in the forelimbs; 4, clonic convulsions in the forelimbs with rearing; and 5, generalized clonic convulsions and loss of balance (27).

2.2. Histological studies

The rats were deeply anesthetized with ketamine (150 mg/kg) and perfused through the ascending aorta with 50-75 ml of heparinised normal saline followed by 75-100 ml of sulfide solution (containing 1.2% Na2S and 1.0% NaH2PO4) and then with 100-125 ml of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following perfusion, the brains removed from the skull, hippocampal blocks were prepared and immersed in 30% sucrose in PB at 4 °C for 1-2 days. Then, sections were cut at a thickness of 20 µm on a freezing microtome (Leica, Germany) and collected in phosphate buffer (0.1 M). Every second section was Nissl-stained with 0.1% cresyl violet (Sigma) and alternate sections were used for Timm staining. In Nissl-stained sections, neuronal loss was quantified in CA1, CA3, and CA4 regions of the hippocampus in at least three sections at a level range between -3.6 and -4.3 mm from the bregma using an image capturing and analysis system (Bel Engineering, Italy). The process was repeated at least two times for each section and its average was taken as the final value. Counting was done blind to the treatments.

To visualize mossy fiber sprouting (MFS) in the inner molecular layer of the dentate gyrus (DG) that accompanies epileptogenesis, we employed a modified Timm histological procedure to label the zinc-containing axons of the granule cells (28). The slices were immersed for 2 min in 100% alcohol, 2 min in 70% alcohol, and 10 min in distilled water. The slices were then developed in the dark under continuous agitation for 60 min in Timm working solution with the following composition: 60 ml of 50% gum Arabic, 10 ml of 2 M sodium citrate buffer (pH 3.7), 30 ml of 5.6% hydroquinone, and 0.5 ml of 17% silver nitrate solution. The staining process was terminated with 2% sodium acetate and the unreacted silver ions were removed with 5% sodium thiosulphate. The sections were then dehydrated and coverslipped. Assessment of MFS (as Timm index) was obtained from the absolute value of the total area of Timm granules divided by the total length of DG (29). The Timm index for each animal was the mean of three sections. All procedures and analyses were done blind to the treatments.

2.3. Statistical analysis

All statistical analysis was performed using SigmaStat software (version 3.5). Values were expressed as means ± SEM. To compare the experimental groups, one-way ANOVA followed by Tukey's *post-hoc* test was used. In all analyses, the null hypothesis was rejected at a level of 0.05.

3. Results

3.1. Seizure activity and behavior

Sham and sham+silymarin groups showed no signs of seizure activity during the first 24 h post-surgery. In contrast, all rats (100%) in kainate group exhibited high scores of seizures. In addition, rats injected with KA and pretreated with silyamrin also exhibited about the same seizure scoresas compared to kainate group (Figure 1).

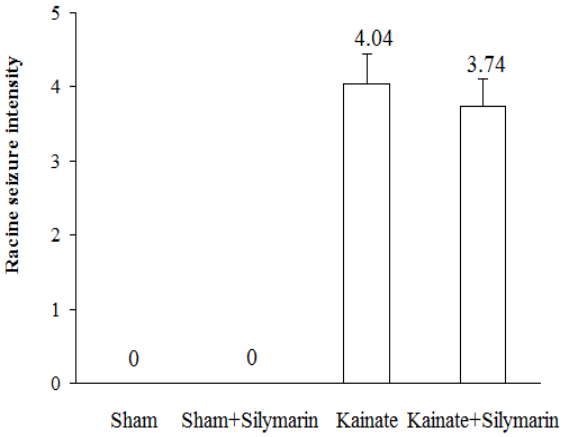

Figure 1. Averaged seizure intensity according to Racine classification in experimental groups.

3.2. Nissl staining findings

In this study, the number of neurons per unit area in the CA1, CA3, and CA4 regions was counted and compared between the groups (Fig. 2). Our results showed that silyamrin pretreatment of sham group did not produce a significant change in this respect. In contrast, intrahippocampal kainate induced a significant degeneration and reduction of neurons in CA3 ($p<0.05$) and CA4 ($p<0.05$) regions of the hippocampus versus sham group. In this regard, the neurodegeneration in the hippocampus was marked by a cell loss in the dentate hilus and considerable thinning of cell layers in the CA3 and CA4 regions and the dentate gyrus of the kainate group typically showed granule cell dispersion and displacement as compared to the contralateral side (non-injected side) in the upper border. Furthermore, silyamrin pretreatment of kainate group did not significantly attenuate these changes in CA3 and CA4 regions as compared to kainate group. These data suggest that silymarin pretreatment could not protect and rescue the neurons of hippocampal regions againast kainate neurotoxicity.

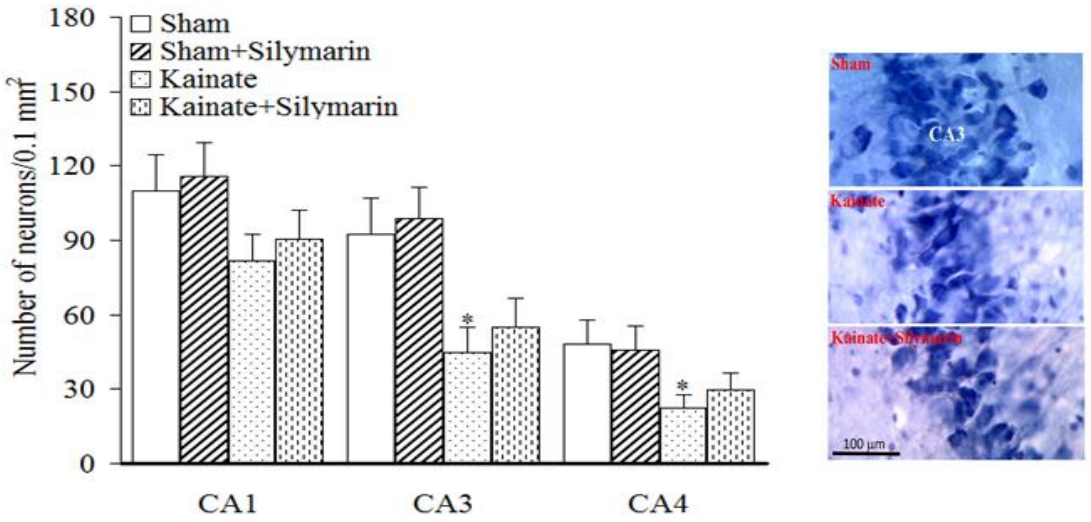

Fig. 2. Number of Nissl-stained neurons in different areas of hippocampus (left panel) and a photomicrograph of coronal sections through the CA3 area of the hippocampus (right panel). * $p<0.05$ (in comparison with Sham) (n=4-5)

3.3. Timm histochemistry

Kainate-induced aberrant MFS was shown by Timm method at 6th week post-lesion that selectively labeled synaptic terminals of mossy fibers due to their high zinc content. In the sham groups, little sprouting was present in the dentate molecular layer. On the contrary, in the kainate group, Timm staining showed robust MFS that extended into the dentate supragranular layer and in the curcumin-pretreated group, supragranular MFS was less intense and more dispersed, though it was still denser than the sham group. We further compared the average width and Timm staining density (as indicated by Timm index) between kainate and silymarin-pretreated kainate groups and found that silymarin treatment could not significantly lower MFS width and staining density. These data indicate that silyamrin could not prevent kainate-induced MFS (Fig. 3).

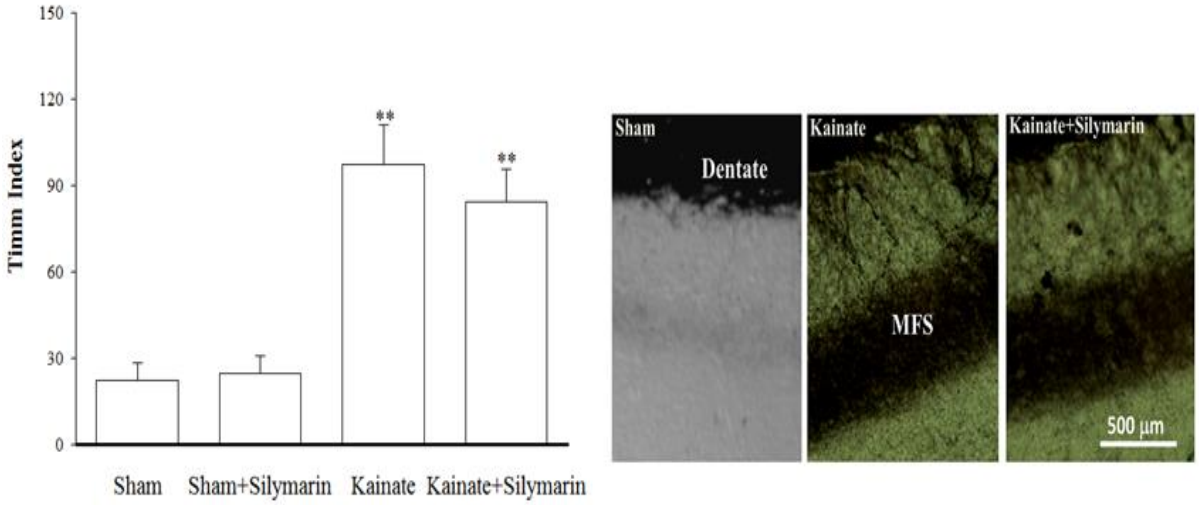

Fig. 3. Timm index as an indicator of mossy fiber sprouting (MFS) (left panel) and a photomicrograph through the hippocampus (dentate region), which shows MFS in Timm staining (right panel) in different groups. (n=4-5) ** $p< 0.01$ (in comparison with Sham)

4. Discussion

TLE is regarded a chronic and resistant to therapy neurological disease with recurrent seizures as a result of development of excitatory and/or inhibitory circuits. Recurrent excitation and the development of seizures have been related to MFS in the hippocampus (30). Intracerebral injection of kainate into CA3 region of the hippocampus leads to development of epileptic seizures. These seizures are associated with a pattern of cell loss that is very similar to that observed in TLE patients (31). For this reason, kainate-induced brain lesion has been generally used for modeling TLE and some excitotoxic neurodegenerative diseases (32). Research evidences indicate that hippocampal oxidative stress is involved in kainate-induced neurotoxicity (33). In this study, a massive neuronal loss was found out in CA3 and CA4 regions in the kainate group. Also, aberrant MFS invading into the granule cell layer and granule cell inner molecular layer in the hippocampus was also observed in kianate group which was consistent with previous studies (29). Kainate injection into the hippocampus led to the degeneration of CA3 pyramidal neurons and dentate hilar cells. The granule cell axons (known as mossy fibers) coming from the dentate lose their postsynaptic targets and sprout into the inner molecular layer. These pathologic changes leads to the formation of a functional recurrent excitatory circuit between granule cells that leads to recurrent seizures (29, 34). In our study, silyamrin at a dose of 100 mg/kg was not capable to lower kainite-induced neurodegeneration in CA3 and CA4 regions of the hippocampus, clearly showing that this compound does not exert a neuroprotective effect in this model of TLE. In addition, this compound was not capable to significantly reduce MFS in kainate group. The main reasons for this issue may be attributed to its dose and administration duration. However, more studies in future are warranted to determine its possible beneficial effect in epilepsy.

In conclusion, SM pretreatment does not reduce seizure intensity, could not preserve hippocampal neurons, and could not lower mossy fiber sprouting in kainate-induced epileptic animals.

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Declaration of interest

There is no conflict of interest for this paper.

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